

AN EVALUATION OF TECHNIQUES USED FOR EXTRACTING ENDOGENOUS CYTOKININS FROM PLANT MATERIAL

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ABSTRACT

Using paper and column chromatography it was found that considerable amounts of cytokinins can be lost from aqueous plant extracts during purification by solvent partitioning. Most, if not all, of the free base cytokinins present in seed extracts of *Rumex obtusifolius* partitioned into petroleum ether at pH 9.0 and ethyl acetate at pH 2.5. Whether the presence of these compounds will be detected by paper chromatography depends very much on the solvent system used.

UITTREKSEL

'N EVALUASIE VAN TEGNIEKE OM SITOKINIENE UIT PLANTMATERIAAL TE EKSTRAHEER.

Met behulp van papier- en kolomchromatografie is gevind dat wanneer plantekstrakte met behulp van organiese oplosmiddels gesuiwer word, relatief groot hoeveelhede van die sitokiniene verlore gaan. Die meeste van die vrye basis sitokiniene in saadekstrakte van *Rumex obtusifolius* beweeg byvoorbeeld in petroleumeter, pH 9,0, en etielasetaat, pH 2,5, in. Of die teenwoordigheid van sodanige verbindings met behulp van papierchromatografie aangetoon sal word, hang grotendeels af van die chromatografiese oplosmiddels wat gebruik word.

INTRODUCTION

The endogenous cytokinins extracted from plant material with aqueous methanol or ethanol are usually purified to some degree prior to being bio-assayed. This is frequently done by partitioning the extracted substances with one or more organic solvents at different pH values.

Pigments and fatty substances have been removed from aqueous plant extracts, at pH 9.0, by partitioning with petroleum ether (Gazit and Blumenfeld, 1970; Prakash and Maheshwari, 1970). These authors could detect no cytokinin activity in the petroleum ether fraction. However, Van Staden and Wareing (1972) have reported cytokinin activity in such extracts. As a number of synthetic cytokinins did not partition into petroleum ether at pH 9.0 it was, at the time, suggested that the activity was perhaps due to substances that are less polar than the substituted adenines which constitute the known cytokinins. This however, does not mean that the natural cytokinins would behave similarly.

In order to remove acidic inhibitors, gibberellins and auxins from plant extracts the pH values of these extracts are adjusted to 2.5 before partitioning against ethyl acetate. That no cytokinin activity could be detected in the ethyl acetate fraction (Gazit and Blumenfeld, 1970; Mayak and Halevy, 1970; Van Staden and Wareing, 1972) is perhaps not surprising as they will contain phenolic compounds as well as inhibitors that would have an adverse effect in most

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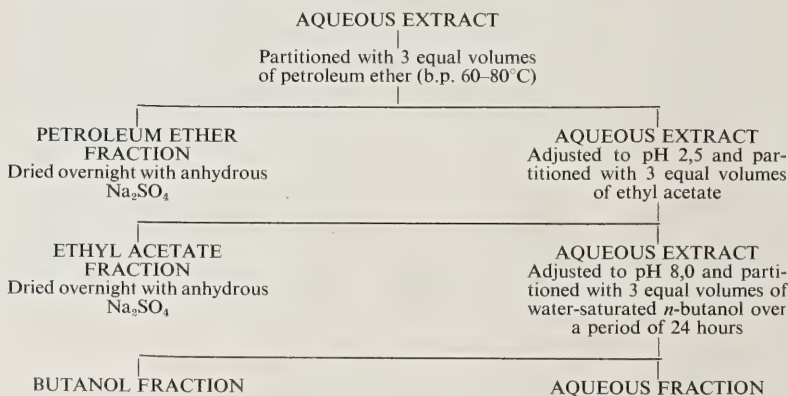
bioassays. In many laboratories ethyl acetate is used routinely for the extraction of cytokinins from aqueous extracts at high pH values; 8.2–8.6 (Upper, Helgeson, Kemp and Schmidt, 1970; Phillips and Cleland, 1972). Thus it is quite possible that even at lower pH values considerable quantities of cytokinin could move into ethyl acetate, but their activity would be masked by interfering compounds.

Following the above partitionings the cytokinins are frequently separated into the butanol-soluble free bases and nucleosides, and the water-soluble nucleotides by partitioning against water-saturated *n*-butanol. It is significant that considerable amounts of butanol-soluble cytokinins may remain in the aqueous extract (Tegley, Witham and Krasnuk, 1971). Their presence could be the result of incomplete partitioning or breakdown of the remaining nucleotides.

The present investigation was an attempt to establish to what extent cytokinins are lost during solvent partitioning, and whether the masking effect of impurities can be eliminated by using different chromatographic solvent systems. Although the soybean callus assay is probably one of the most specific and reliable (Letham, 1967), different results might be obtained when other bioassays are used.

MATERIAL AND METHODS

Ten gram samples of seed of *Rumex obtusifolius* L. were incubated at 20°C on moist filter paper in the dark for 48 hours. They were then exposed to 15 minutes red light and incubated for a further two days in the dark. At the end of this period the seed was ground in 500 ml 80% ethanol and extracted for 24 hours at 4°C and then filtered. The residue was washed with a further 250 ml ethanol and then discarded. The combined filtrates were reduced to the aqueous phase, brought to 100 ml, adjusted to pH 9.0 and extracted as follows:



Only redistilled solvents were used for partitioning. All fractions were taken to dryness under vacuum at 35°C, redissolved in 80% ethanol and streaked on Whatman No. 3MM chromatography paper and developed with solvent system A, viz., water-saturated *sec*-butanol. In addition, the following solvent systems were employed: B, *sec*-butanol: 25% ammonium hydroxide (4:1 v/v); C, *iso*-propanol:ammonia:water (10:1:1 v/v). Each chromatogram was thoroughly air dried and divided into 10 equal R_f strips. Each strip was assayed separately for cytokinin activity using the soybean callus bioassay. The different fractions were also fractionated on a Sephadex LH-20 column (2,5 × 90 cm) as described by Armstrong, Burrows, Evans and Skoog (1969). The cytokinins were eluted with 35% redistilled ethanol at a flow rate of 20 ml/hour. Forty millilitre fractions were evaporated to dryness at 30°C and assayed for cytokinin activity. Three callus explants were transferred to each flask and maintained at 25 ± 3°C under continuous illumination for 28 days. The values presented in the figures are the means of the separate extractions and bioassays.

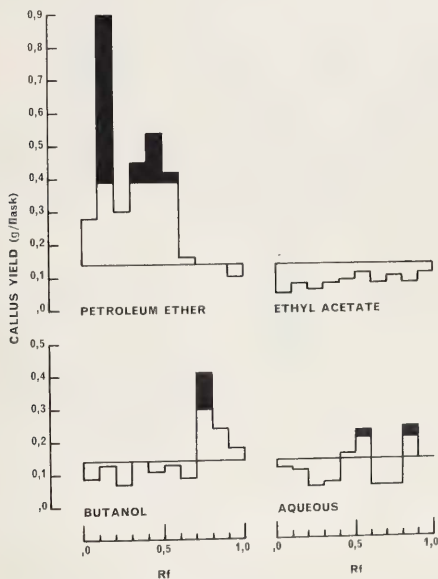


FIG. 1.

Histograms of soybean callus bioassays of extracts from ten grams dry weight equivalent of *Rumex* seed after paper chromatography in water-saturated *sec*-butanol. The black areas represents activity statistically significant from controls at the one per cent level.

RESULTS AND DISCUSSION

When the plant extracts obtained from *Rumex* seed were separated in solvent system A most cytokinin activity was evident in the petroleum ether fraction (Fig. 1). This, however, may not be a true reflection, as the petroleum ether fraction is relatively "clean" compared with the ethyl acetate, butanol and aqueous fractions which contained large amounts of phenolics and other impurities. Furthermore, these impurities have the tendency to streak over most of the chromatogram when water-saturated *sec*-butanol is used. It is thus quite possible that the cytokinin levels in the ethyl acetate, butanol and aqueous fractions are higher than indicated by the bioassays (Fig. 1). That this is actually the case was shown, when most of the impurities were eliminated by using other chromatographic solvent systems. When solvent systems B and C were used it became apparent that the butanol fraction contained a higher concentration of cytokinin than the petroleum ether fraction (Figs. 2 and 3). This was further substantiated when the extracts were fractionated on a Sephadex LH-20 column (Fig. 4). Elimination of interfering substances also revealed that rela-

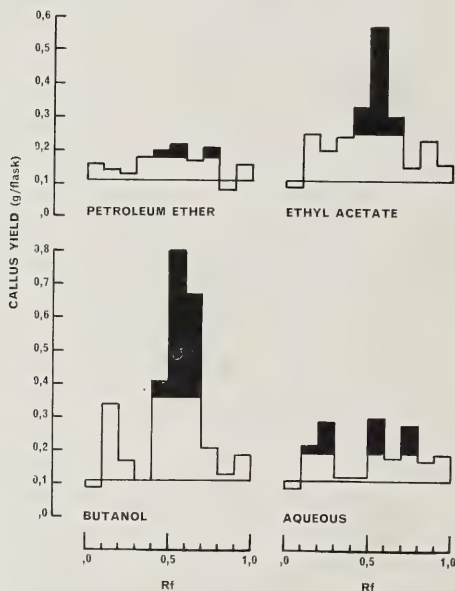


FIG. 2.

Histograms of soybean callus bioassays of extracts from ten grams dry weight equivalent of *Rumex* seed after paper chromatography in *sec*-butanol: 25% ammonium hydroxide (4:1 v/v). The black areas represents activity statistically significant from controls at the one per cent level.

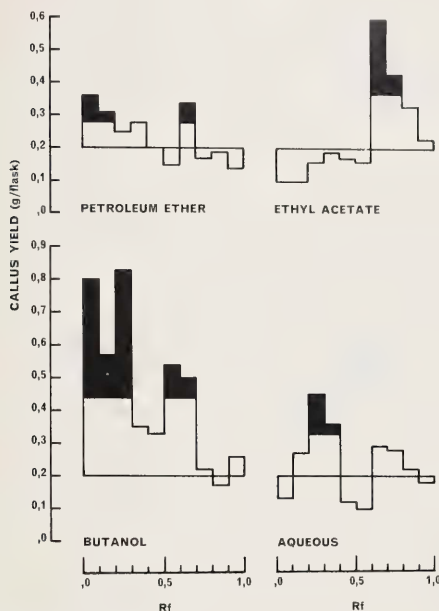


FIG. 3.

Histograms of soybean callus bioassays of extracts from ten grams dry weight equivalent of *Rumex* seed after paper chromatography in *iso*-propanol: ammonia: water (10:1:1 v/v). The black areas represents activity statistically significant from controls at the one per cent level.

tively large amounts of cytokinin were present in the acidic ethyl acetate fraction, which showed no signs of activity when separated with solvent A. These results clearly demonstrate that failure to detect cytokinin activity may be due to incomplete removal of interfering substances.

By fractionating plant extracts on a Sephadex LH-20 column Armstrong *et al.* (1969) were able to separate free base and nucleoside cytokinins. The present results obtained with paper chromatography indicate that cytokinins can partition into all of the solvents used. However, as the free bases and nucleosides do not separate clearly from one another in the solvent systems, no information is available as to the possible nature of the cytokinins involved. The different fractions were therefore separated by column chromatography. This yields more information about the nature of the compounds and excludes the toxic effect of residual alcohols and/or ammonia from the solvent systems in the bioassay. The results in Figure 4 also demonstrate that cytokinins were present in all fractions. Of particular significance is the fact that the major

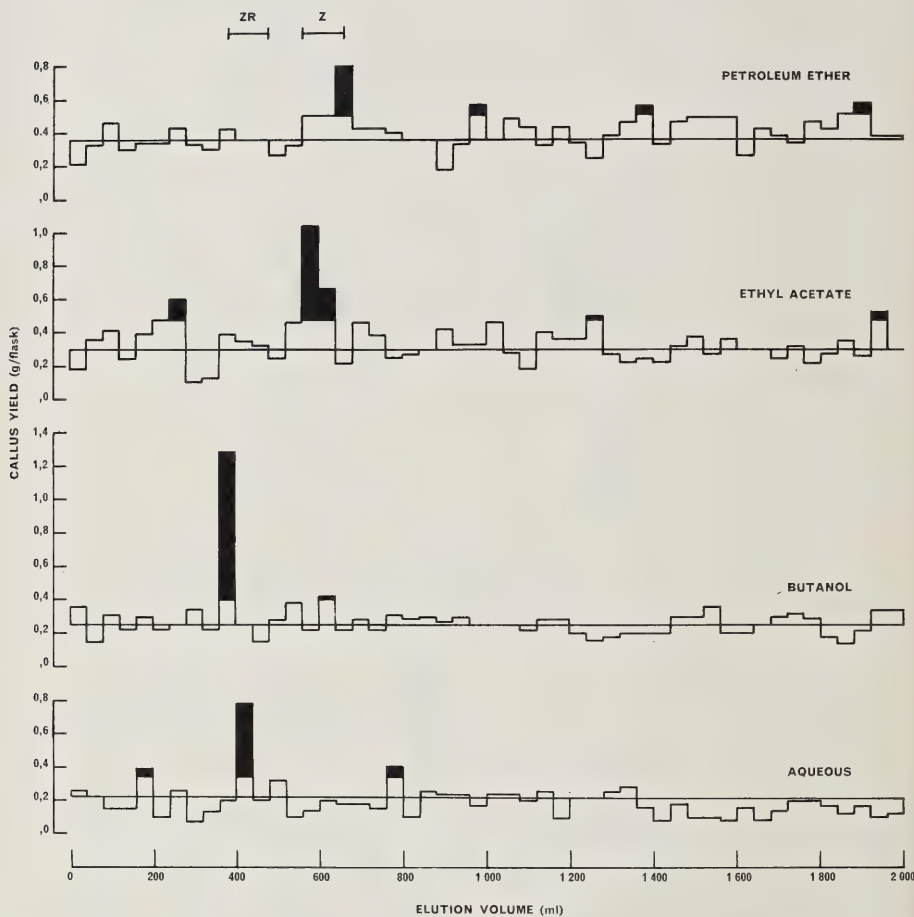


FIG. 4.

The distribution of cytokinin activity of a Sephadex LH-20 fractionation of extracts from ten grams dry weight equivalent of *Rumex* seed. The column was eluted with 35% ethanol at a flow rate of 20 ml/hour. The black areas represents activity statistically significant from controls at the one per cent level. Z = zeatin, ZR = zeatin riboside.

peak of activity in the petroleum ether and ethyl acetate fractions co-chromatographed with zeatin. Whilst it is appreciated that this cannot be regarded as proof of identity it suggests that free bases will readily partition into these solvents. The major peak of cytokinin activity in the butanol and aqueous fractions co-chromatographed with zeatin riboside. Very little activity was found in these fractions in that region where zeatin normally elutes. The presence of cytokinin activity in the aqueous fraction that co-chromatographed with zeatin riboside could result from incomplete partitioning against butanol, even after 24 hours. Alternatively the nucleotide could have been hydrolyzed to its corresponding nucleoside.

From the data presented it is evident that considerable amounts of cytokinin can be lost during purification by partitioning. In fact most of the free base cytokinins in the seed extracts appeared to have been lost by partitioning against petroleum ether and ethyl acetate (Fig. 4). If these fractions are not properly screened for cytokinin activity one could easily be led to believe that only nucleosides were present in the plant extracts. If the plant extracts under investigation are reasonably pure it would probably be advisable to eliminate solvent partitioning to an absolute minimum.

When the primary object of the investigation is to identify endogenous cytokinins these losses are probably not serious although inconclusive results would be obtained if the cytokinin concentration is low. However, in studies where the possible participation of endogenous cytokinins in physiological phenomena is investigated the losses are significant.

ACKNOWLEDGEMENTS

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